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TITLE: Role of APOE Isoforms in the Pathogenesis of TBI induced Alzheimer's Disease

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14. ABSTRACT During the reported period, we completed the behavioral tests with *Abca1* deficient mice. Three behavioral paradigms were used to evaluate the differences in cognitive performance between the genotypes and age groups following TBI: the analysis of the data generated in the Elevated Plus Maze paradigm demonstrated significant increase in anxiety related behavior with a difference between sham and injured WT or *Abca1* deficient mice expressing APOE4. In the MWM paradigm, both *Abca1* deficient genotypes had performance significantly affected by training and genotype/injury, and the analysis suggested a negative effect of *Abca1* deficiency in mice expressing either isoform. TBI had no effect on performance of fear conditioning in *Abca1* deficient mice regardless of the expressed APOE isoform. Targeted RT-QPCR for genes involved in inflammatory response, demonstrated that the injury affects transcriptional activity beyond the adjacent tissue and likely the entire brain. We continued the analysis of the changes in the transcriptome of young and aged APOE3 and APOE4 mice (expressing two copies of *Abca1*) with the goal to identify co-expression gene networks correlated to the traits (age / injury / genotype) in response to TBI. The results clearly demonstrate segregation by injury status and separation by APOE isoform, although not as strong as expected. Most importantly the networks identified by correlation analysis corresponded to GO terms "immune response", "innate immunity" with differential expression of genes involved in pattern recognition and phagocytosis. The results of the study at this stage have been presented at three scientific meetings and a manuscript is being submitted.

15. SUBJECT TERMS
Abca1 global deletion, APOE targeted replacement, complex breeding, CCI model optimization, mRNA library generation, high throughput massive parallel sequencing, analysis

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1. **INTRODUCTION:**
   - Patients carrying apolipoprotein E4 (APOE) allele are more susceptible to poor neurological outcome after traumatic brain injury (TBI). Futhermore, the inheritance of APOE4 is the only proven genetic risk factor for sporadic Alzheimer's disease (AD). Importantly, TBI is a risk factor for the subsequent development of AD, particularly among APOE4 carriers. ATP binding cassette transporter A1 (ABCA1) is a lipid transporter that controls the generation of HDL in plasma and ApoE-containing lipoproteins in the brain which are important for the repairing of axonal damage after TBI. We demonstrate that the lack of Abca1 increases amyloid plaques and decreased APOE protein levels in AD-model mice. In this proposal we will test the hypothesis that ABCA1 differentially affects the response of mice expressing human APOE isoforms to TBI. We will evaluate the immediate as well as the long-term effects following brain injury.
2. **KEYWORDS:**
   - Traumatic brain injury, APOE isoforms, ABCA1, Alzheimer's disease, APP mice, amyloid beta, axonal injury, inflammatory reaction, transcriptome, high throughput massive parallel sequencing, mRNA-seq., behavioral testing, memory impairment, recovery
3. **ACCOMPLISHMENTS:**
   - **What were the major goals of the project?**
     - The Aims are: 1) To determine the effect of Abca1 deficiency on the response to TBI in mice expressing human APOE3 and APOE4 isoforms, and 2) To evaluate the development of AD-like phenotype in APP expressing mice exposed to TBI and to determine if the differences in phenotype are mediated through ABCA1.
   - **What was accomplished under these goals?**

Methods

Animals

All animal experiments were approved through the University of Pittsburgh Institutional Animal Care and Use Committee and carried out in accordance with PHS policies on the use of animals in research. We used human APOE4$^{+/+}$ and APOE3$^{+/+}$ targeted replacement mice on a C57BL/6 background (Fitz et al., 2012). Additionally, these mice were crossed with *Abca1*$^{-/-}$ to produce APOE3$^{+/+}$,*Abca*$^{+/-}$ and APOE4$^{+/+}$,*Abca*$^{+/-}$, which underwent all the same procedures as their *Abca1*$^{+/+}$ counterparts. Experimental male and female APOE3 or APOE4 mice were kept on a 12 h light-dark cycle with *ad libitum* access to food and water. Mice were randomly assigned to either sham or controlled cortical impact (CCI) experimental group and handled for 2 days (5 min per day) starting at 3 mo of age. Following surgical procedures, mice were allowed to recover for 3 days before starting behavioral testing. All materials were purchased through Thermo Fisher Scientific, unless otherwise noted.

Controlled Cortical Impact

CCI model of brain injury was performed according to previous published methods (Brody et al., 2007). Following induction of anesthesia with 5% isoflurane, the mouse was moved to the stereotaxic frame, where the head was secured, core body temperature maintained at 37°C using a heating pad and anesthesia continued with 1.5% isoflurane. The head was shaven, surgical site sterilized with two separate iodine - alcohol washes, a 50% mixture of bupivacaine and lidocaine applied to the surgical site and ophthalmic ointment applied to the eyes. The scalp was opened with a midline incision exposing the dorsal aspect of the skull and the skull leveled. A 4.5 mm diameter craniotomy was performed over the left parietal cortex using a dental drill. Once the bone flap was removed, mice in the CCI group received a single impact at 1.0 mm depth with a 3.0 mm diameter metal tip onto the cortex (3 m/s, 100 ms dwell time; Impact One, Leica). Sham mice received identical anesthesia and craniotomy, but did not receive impact and are considered negative controls. Following the impact, the surgical site was sutured, triple antibiotic cream applied, Buprenex (0.1 mg/kg; IP) provided for analgesia, and sterile saline administered for rehydration. Mice were allowed to recover on heating pad, until freely mobile, before returning to their home cage.

Elevated-Plus Maze

The elevated plus maze (EPM, San Diego Instruments) test was performed 4 days post-injury as described previously (Washington et al., 2012). The maze consists of 4 arms in the shape of a "+". All arms are the same length (30.5 cm) with a central square (10x10 cm); 2 arms are open on the sides, and 2 have 16 cm high walls. The entire maze is raised 40 cm off the ground. The elevated plus maze tests anxiety-related behavior by utilizing rodent's fear of open and elevated spaces. Mice are placed into the maze within the center square facing a closed arm and are allowed to explore for 5 min. Percent time spent in each arm was tracked using the ANYMaze software (Stoelting Co.) from a camera positioned over the maze. 50% of body area within an arm was established in ANYMaze for definition of entry.

Morris Water Maze

Spatial navigational learning and memory retention were assessed using Morris water maze (MWM) as described previously (Fitz et al., 2010; Lefterov et al., 2010); with testing performed on days 6-12 post-injury. Briefly, in a circular pool of water (diameter 122 cm, height 51 cm, temperature 21 ± 1°C), we measured the ability of mice to form a spatial relationship between a safe but invisible platform (submerged 1 cm below the water level; 10 cm in diameter) and several visual extra maze cues surrounding the pool of water. On day 6 post-injury, mice received a habituation trial, during which the animals were allowed to explore the pool of water without the platform present. Beginning the next day, they received four daily hidden platform training (acquisition) trials with 10–12-min inter-trial intervals for five consecutive days (days 7-11 post-injury). The platform remained in the center of one of the four quadrants of the pool (target quadrant). Animals were allowed 60 s to locate the platform and 20 s to

remain there. Mice that failed to find the platform were lead to the platform by the experimenter and allowed to rest there for 20 s. Performance was recorded using AnyMaze software (Stoelting Co.) during all trials. During the acquisition trials, escape latency (time to reach the platform) was subsequently used to analyze and compare the performance between all groups.

Contextual Fear Conditioning

Contextual fear conditioning test was performed on days 13 and 14 post-injury as described previously (Kornecook *et al.*, 2010) with minor modifications. Briefly, mice were placed in a conditioning chamber for 2 min before receiving a 2 s, 0.7 mA footshock through the bars on the floor of the chamber, and this cycle was repeated once more. The mice were allowed to remain in the chamber for 60 s before being returned to their housing cages. Contextual fear was evaluated 24 h after training by measuring freezing behavior during a 5 min time period in the original chamber before mice were returned to their housing cages. Freezing behavior, defined as the absence of movement except for that needed for breathing, was scored using AnyMaze software. All chambers were cleaned between animals with 70% ethanol. Data are represented as percentage freezing.

Animal Tissue Processing

Fourteen days post-injury, mice were anesthetized using Avertin (250 mg/kg of body weight, i.p.) and perfused transcardially with 20mL of cold 0.1M PBS pH 7.4, following a blood draw from the right atrium (Nam et al., 2016). Brains were rapidly removed and a 1.5 mm coronal section of the brain, including the injury site, was taken by slicing the brain at -2.5 mm and -4.0 from bregma. Within the coronal slice, the hemispheres were separated, and the subcortical tissue was dissected out; hippocampal and cortical tissue were snap-frozen together for RNA-seq and RT-qPCR analysis. The remaining anterior of the brain was fixed in formalin for immunhistochemisty.

RNA isolation and mRNA sequencing

All procedures were performed as before (Nam et al., 2016). Four APOE3 and APOE4 male and female mice per sham and CCI injured group were used for RNA-seq. RNA was isolated from frozen cortices and hippocampi at the injury site and purified using RNeasy kit (Qiagen) according to the manufacturer recommendations. Quality control of all RNA samples was performed on a 2100 Bioanalyzer instrument and samples with RIN > 8 were further used for library construction using mRNA Library Prep Reagent Set (Illumina). Libraries were generated by PCR enrichment including incorporation of barcodes to enable multiplexing. The libraries, were sequenced on Illumina HiSeq2000. For RT-qPCR, first strand cDNA was synthesized from 1 μg of total RNA using EcoDry™ Premix, Random Hexamers (Clontech). Next Generation Sequencing of libraries was performed by the Next Generation Sequencing Center (University of Pennsylvania) on *HiSeq 2500* machines. Following initial processing and quality control at the Center, the sequencing datasets as **Fastq** files were downloaded and further analyzed. For read alignment, we used Subread aligners. Differential gene expression, in all cases, is calculated using **edgeR**

statistical package on the **Bioconductor**. Lists of differentially expressed genes are further analyzed as described in the following section.

Functional Pathway analysis

We performed functional annotation clustering using the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/version6.7) (Ficenec et al., 2003)and Gene set enrichment Analysis (GSEA v2.2.2, https://www.broadinstitute.org/GSEA) (Mootha et al., 2003; Subramanian et al., 2005).

Weighted Gene Co-expression Network Analysis

The analysis was performed with version v.1.49 of WGCNA (Zhao et al., 2010). A raw count exclusion was performed where any gene expression average <5 reads per million are discarded to eliminate noise. The program then clusters the animals by gene expression enabling the detection of outliers. A scale free topology model was applied to the data, determining the power. Modules were generated automatically using a soft thresholding power, $\beta=10$, and definition parameters included a minimum module size of 33 genes and a minimum module merge cut height of 0.25. Modules were named by conventional color scheme and then correlated with trait data (ApoE isoform, Injury). Statistical significance was determined by student's t-test, $p<0.05$.

All the modules were summarized by module eigengenes (ME), the first principle component of each module that was calculated as a synthetic gene representing the expression profile of all the genes within a given module.

Statistical Analyses

All results are reported as means ± S.E.M. To determine statistical significance between groups in EPM, MWM and FC, we used two-way ANOVA with a Tukey *post hoc* test. Unless otherwise indicated, all statistical analyses were performed in GraphPad Prism, version 7.0 (La Jolla, CA) and differences were considered significant where $p<0.05$.

Results

Changes in anxiety related behavior following TBI

TBI causes significant increase in percent time spent in open-arms of the elevated plus maze of Abca1 deficient mice (Figure 1; $p<0.0001$). The increase is significant between sham and TBI animals among E4 animals, both Abca1 deficient wildtype and heterozygous, again, continuing the trend of E4 animals having a higher impact of TBI at acute time-points.

TBI causes cognitive impairments as measured in MWM performance

TBI continues to cause cognitive impairments as seen in performance of Morris Water maze among Abca1 deficient animals (Figure 2). Both Abca1 deficient genotypes had performance significantly affected by training ($p<0.0001$) and genotype/injury ($p<0.0001$), similar to what we had seen

in young E3 and E4 Abca1 wildtype animals. Again, there was no interaction between training day and genotype/injury among Abca1 deficient animals only, however, in comparing Abca1 wildtype and deficient animals on the E3 background only, there was an interaction (Figure 3, $p<0.05$). This suggests that Abca1 deficiency in addition to the expression of apolipoprotein E3 has an even higher impact following traumatic brain injury.

Changes in Contextual Fear Conditioning.

Genotype continues to impact cognitive performance in fear conditioning among Abca1 deficient animals (Figure 4). Consistent with previous results in Abca1 wildtype animals, traumatic brain injury has no effect on performance of fear conditioning, even in animals heterozygous for Abca1 expression. Alternatively, genotype still continues to affect freezing response in this test ($p<0.05$).

Targeted RT-QPCR to evaluate changes in gene expression

We performed RT-QPCR expression assays for Trem2 and Abca1. Recent discoveries have indicated that Trem2 may be associated with high risk of late onset Alzheimer's disease. Following traumatic brain injury, gene expression of Trem2 was increased both in the ipsilateral and contralateral hemispheres of the brain (Figure 5). Similar results are seen for Abca1 (Figure 6). These results suggest that the injury affects transcriptional activity beyond the adjacent tissue, largely impacting the whole brain.

Generation of co-expression networks and trait correlation

WGCNA was first applied to identify similarities between the samples (libraries/animals) so they were clustered as shown on Figure 7. We did not have any outlying samples and none of the animals were excluded from the downstream analysis. In addition to the clustering, the corresponding traits, as initially defined, are visualized at the bottom of the dendrogram. The samples were clearly segregated by injury status and by age, whereas separation by APOE isoform was not as strong.

Expression profiles of hippocampi and cortices from E3 and E4 mice with and without injury, at 3 months or 9 months of age, were used to generate co-expression networks. In the co-expression networks, we were most interested in modules that were significantly differentially expressed between conditions, age (young vs adults), genotype (E3 vs. E4), and treatment (sham vs. TBI): (Table 1). Brown and magenta modules in TBI and red and turquoise in Aging were upregulated, while purple in TBI and yellow and black in aging were down-regulated. Blue and green were significantly down-regulated modules across both TBI and Aging. WGCNA further identified the overlap between modules, which was visualized as a heatmap using only the top 500 expressed gene (Figure 8) The Brown module, where the consensus for gene expression is up-regulated, is the most significantly related to TBI. The expression levels for all genes in the network are shown for each sample (Figure 9) with the corresponding ME displayed below. Using the genes within the module, the modules were annotated using **Kyoto Encyclopedia of Genes and Genomes** (KEGG) and Gene Ontology enrichment analysis. These results

implicated the genes in the brown module as being the most strongly enriched for GO terms “Immune response” and “inflammatory response”. KEGG pathways significantly enriched in the brown module were “lysosome”, “Toll-like receptor signaling pathway”, and “apoptosis”.

Our next goal was to identify the most important genes within the network. The most highly connected genes within the module network are called Hub genes. The hub genes were identified by their Module Membership (MM) value and their intramodular connectivity (Figure 10). Any gene with a higher MM value than 0.8 was identified as a hub gene. In the brown network these genes are also the highest interconnected genes when MM is correlated to intramodular connectivity. For the brown module we identified 51 genes as hub genes. We were mostly interested in hub genes that corresponded to the identified function of the module and have been shown to be involved in TBI in the literature. In the Brown module, the hub genes of interest were *Tyrobp, Clu* and *Axl*. Cytoscape is an open source software platform for visualizing complex networks and integrating these with any type of attribute data. We used Cytoscape and built a network based on the hub genes and their targeted genes that corresponded to the overall module function (Figure 11).

To further identify enriched pathways commonly affected by TBI in both isoforms, we used Gene Set Enrichment Analysis (GSEA) assessing all transcripts without a cutoff (Subramanian et al., 2005) (Figure 12). To examine the effect of injury on transcriptome, we combined the results for both isoforms in TBI group (E3-TBI+E4-TBI) and compared them to sham treated (E3-sham + E4-sham). GSEA results reveal the top biological process categories upregulated by TBI and amongst them are “Immune System Response”, “Receptor Activity”, “Cysteine Type Endopeptidase Activity”, “G Protein-coupled Receptor Binding” and “Chemokine Receptor Binding”. Top downregulated categories are “Synaptic Transmission” and “Potassium Ion Transport” but they were not statistically significant.

In contrast to ME brown, we found that ME green is negatively associated with injury suggesting that genes members of this module are downregulated by TBI (Figure 13) The related biological process that represents this network (module size = 854) was associated with GO category “transport”. We were most interested in hub genes connected to this network such as calcium/calmodulin-dependent protein kinase 2B (*Camk2b*), and phosphatidylinositol 3-kinase regulatory subunit 2 (*Pik3r2*).

ME Darkred is significantly associated with APOE isoform regardless of injury (Figure 14). The heat-map and bar plot shown on demonstrate an increased expression of the genes associated with this network in all APOE4 mice (TBI+Sham) and an opposite effect in all APOE3 mice (TBI+Sham). The biological process associated with ME darkred network (module size = 31) was the GO term “innate immunity”. We were interested in several hub genes associated with “innate immunity” biological process such as osteoclast-associated immunoglobulin-like receptor (*Oscar*) and proto-oncogene receptor tyrosine kinase *Fyn*, which were used to build a representative network.

ME salmon positively correlated to APOE4 mice and negatively to APOE3 (r=0.34, p=0.0056). Functionally, ME "salmon" (module size = 65) is enriched in genes connected to the biological process "myelination" (Figure 15). The network is significantly enriched on oligodendrocytes signature genes (18 genes out of 64 were oligodendrocytes specific; fold enrichment 30%). We used hub genes myelin basic protein (*Mbp*), myelin regulatory factor (*Myrf*), and proteolipid protein 1 (*Plp1*).

Finally, we have been working to develop an immunohistochemistry protocol against Trem2 using a fluorescent secondary, which would allow us to label against other pathology markers. We used APP/PS1Δ9 mice since the prolonged amyloid pathology would serve as a stimulus for Trem2 expression. Figure 16 shows the successful development of the fluorescent immunohistochemistry protocol with staining for microglia (Iba1), Trem2, and nuclei (DAPI) or Aβ plaque (X34). With the successful development of this protocol, it can now be applied to the TBI study.

- **What opportunities for training and professional development has the project provided?**
  - There have been several opportunities for training and professional development of the graduate student, Emilie Castranio. Ms. Castranio has been working on an individual basis with both Dr. Koldamova and Dr. Lefterov to develop multiple skills. Specifically, Dr. Koldamova has been training Ms. Castranio in western blot protein validation for use on this project, and both Drs. Koldamova and Lefterov have been working with Ms. Castranio to develop writing skills towards the submission of the first manuscript from data produced by this grant. Additionally, Ms. Castranio was able to attend multiple conferences to present her work and receive feedback, as well as attend seminars at these conferences to learn about novel techniques that could be applied to the project and learn about the current status of the field.
- **How were the results disseminated to communities of interest?**
  - Nothing to report
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - For the next reporting period, the behavioral testing for all *Abca1* deficient groups should be completed and analyzed. Additionally, the libraries should be generated and sequenced. The data resulting from sequencing can then undergo integrated analyses, using WGCNA and GSEA, to determine how and the *Abca1* deficiency could modulate the effect of the injury on the transcriptome. Additionally, the lab will continue to develop and optimize Trem2 immunohistochemistry and several western blot validations. Ultimately, we are expecting to submit a manuscript in the next quarter on the *Abca1* wildtype mice and then completely focus on developing a second manuscript on the *Abca1* deficient mice.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**

- This project will provide a better understanding of how inheritance of the ApoE allele modulates the response to injury. Currently, our results reveal that while APOE and injury each have a strong influence on the mouse phenotype, there is no interaction between these two factors. Using WGCNA was imperative to separating the effect of APOE and TBI to show what networks of gene are impacted by each factor. Going forward, we hope to reveal how deficiency in *Abca1* modulates the response to injury and amyloid in TBI mice.

- **What was the impact on other disciplines?**
  - Nothing to report.
- **What was the impact on technology transfer?**
  - Nothing to report.
- **What was the impact on society beyond science and technology?**
  - Nothing to report.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**
  - Nothing to report
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - There has been a delay in generating mRNA-seq libraries for mice on Abca1+/- background. As a result there is an inevitable delay in receiving the sequencing sets for further analysis, even though, thanks to previous decisions of USAMRAA Grants Officers, we perform the analysis of sequencing files in our lab. Technical and time constraints outside our control that inevitably delay the completion of the study within the requested period. This, however, has been resolved through the submission and acceptance of a no cost extension.
- **Changes that had a significant impact on expenditures**
  - Nothing to report

6. **PRODUCTS**

- **Journal publications.**
  - K.N. Nam[+], A. Mounier[+], C.M. Wolfe, N.F. Fitz, F. Letronne, A.Y. Carter, V.L. Reeves, Y. Tyurina, V. Kagan, J. Wang, X. Han, J. Schug, I. Lefterov & R. Koldamova; "Integrated approach reveals diet and APOE isoforms differentially affect immune response in Alzheimer's model mice"; *Sci Tr Med,* 2016; Submitted, under review. Federal support acknowledged.
  - K.N. Nam, A. Mounier, N.F. Fitz, C.M. Wolfe, J. Schug, I. Lefterov, R. Koldamova; "RXR controlled regulatory networks identified in mouse brain counteract deleterious effects of Abeta oligomers"; *Sci Rep* 6, 2016. doi: 10.1038/srep24048. Published. Federal support acknowledged.
  - N.F. Fitz, V. Tapias, A.A. Cronican, E.L. Castranio, M. Saleem, A.Y. Carter, M. Lefterova, I. Lefterov, R. Koldamova. "Opposing effects of Apoe/Apoa1 double deletion on amyloid

pathology and cognitive performance in APP mice"; *Brain*, Dec. 2016. Doi: 10.1093/brain/awv293. Published. Federal support acknowledged.

- A. Mounier[+], D. Georgiev[+], K.N. Nam, N.F. Fitz, E.L. Castranio, C.M. Wolfe, A.A. Cronican, J. Schug, I. Lefterov, R. Koldamova. " Bexarotene-activated retinoid X receptors regulate neuron differentiation and dendritic complexity"; *J Neurosci,*Aug, 2015. Doi: 10.1523/JNEUROSCI.1001-15.2015. Published. Federal support acknowledged.
- I. Lefterov, J. Schug, A. Mounier, K.N. Nam, N.F. Fitz, R. Koldamova. "RNA-sequencing reveals transcriptional up-regulation of Trem2 in response to bexarotene treatment" *Neurobiol Dis,*Oct. 2015. Doi: 10.1016/j.nbd.2015.05.019. Published. Federal support acknowledged.

- **Other publications, conference papers, and presentations.**
  - Poster Presentation: Castranio, E.L., et al. Effect of Age and APOE Isoform on Traumatic Brain Injury in Mice. Presented at Microglia in the Brain (Z4) Keystone Meeting, Organizers: Beth Stevens & Richard M. Ransohoff. Jun 12–16, 2016; Keystone Resort Keystone, Colorado. Figure 17.

  - Poster Presentation: E.L. Castranio[1], A. Mounier[1], C. M. Wolfe[1], J. Schug[2,3], N.F. Fitz[1], R. Koldamova[1], I. Lefterov[1] "Effect of Age and APOE Isoform on Traumatic Brain Injury in Mice". Gordon Research Conference: Neurobiology of Brain Disorders Organizers: Luciano D'Adamio & Bart De Strooper. August 7-12; PGA Catalonia Business and Convention Centre Girona, Spain. Figure 18.

  - Poster Presentation: E.L. Castranio[1], A. Mounier[1], C. M. Wolfe[1], J. Schug[2,3], N.F. Fitz[1], R. Koldamova[1], I. Lefterov[1] "Integrated genomic approaches identify changes in interconnected networks and major pathways following traumatic brain injury in young and aged APOE3 and APOE4 mice". Society for Neuroscience Annual Meeting San Diego, CA. Figure 19.

- **Website(s) or other Internet site(s)**
  - http://koldamovalefterovlab.pitt.edu/index.html
  - The URL listed above is the website of the laboratory. It includes a short description of the research that the Koldamova-Lefterov lab conducts.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	Iliya Lefterov
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-0249-0280
Nearest person month worked:	12
Contribution to Project:	Dr. Lefterov was involved in experimental creation and design, and training of graduate students.
Funding Support:	NIA, NIEHS

Name:	Radosveta Koldamova
Project Role:	Co-PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-6761-0984
Nearest person month worked:	1
Contribution to Project:	Dr. Koldamova was involved in experimental design and training of graduate students.
Funding Support:	NIA, NIEHS

Name:	Nicholas Fitz
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0001-6938-6536
Nearest person month worked:	3
Contribution to Project:	Dr. Fitz has overseen maintenance of the colony and experimental design, as well as quality control.
Funding Support:	NIH, NIA

Name:	Anais Mounier
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	0000-0003-4421-1814
Nearest person month worked:	4
Contribution to Project:	Dr. Mounier performed work in the area of RNA, including isolation from tissue, generation of libraries and RT-qPCR.
Funding Support:	Dr. Mounier left the lab on Aug. 1, 2016 and will no longer be on the personnel list for 2017.

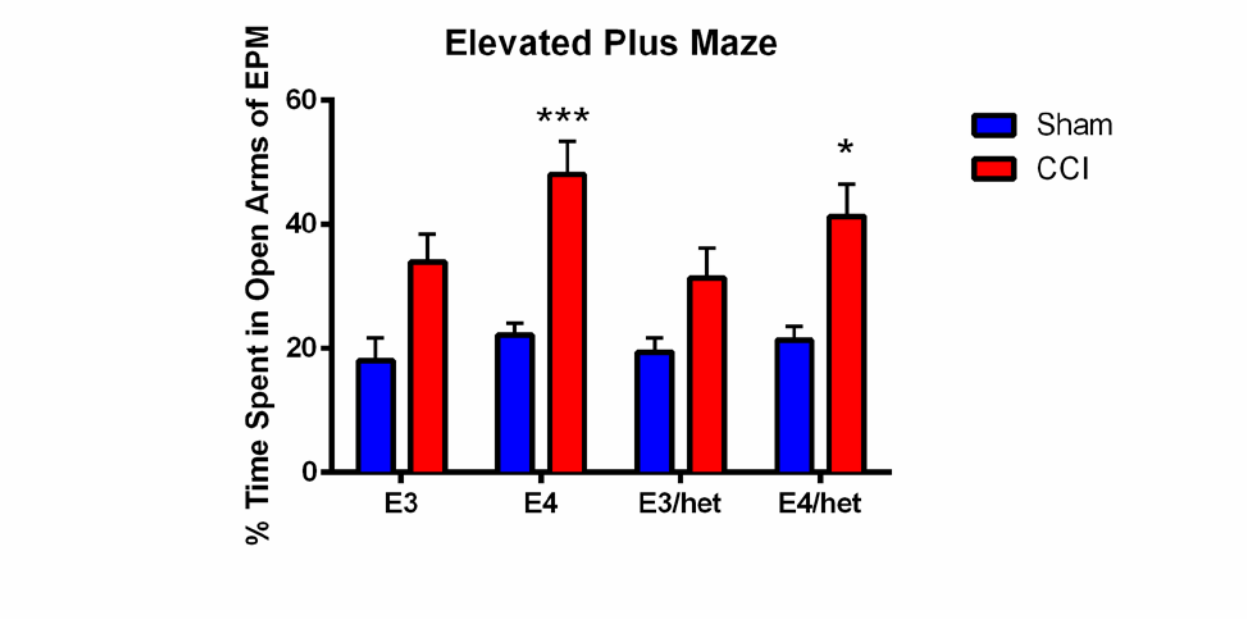
Name:	Florent Letronne
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	0000-0002-5330-0395
Nearest person month worked:	1
Contribution to Project:	Dr. Letronne has taken over Dr. Mounier’s responsibilities concerning biochemistry.
Funding Support:	NIH

Name:	Hafsa Kamboh
Project Role:	Predoctoral fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	5
Contribution to Project:	Dr. Kamboh has performed work in protein validation and colony management.
Funding Support:	NIA

Name:	Emilie Castranio
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	0000-0001-6817-7388
Nearest person month worked:	12
Contribution to Project:	Ms. Castranio has performed work in the area of surgeries, and behavior, tissue analysis, biochemistry, immunohistochemistry and overall analysis.
Funding Support:	NIA

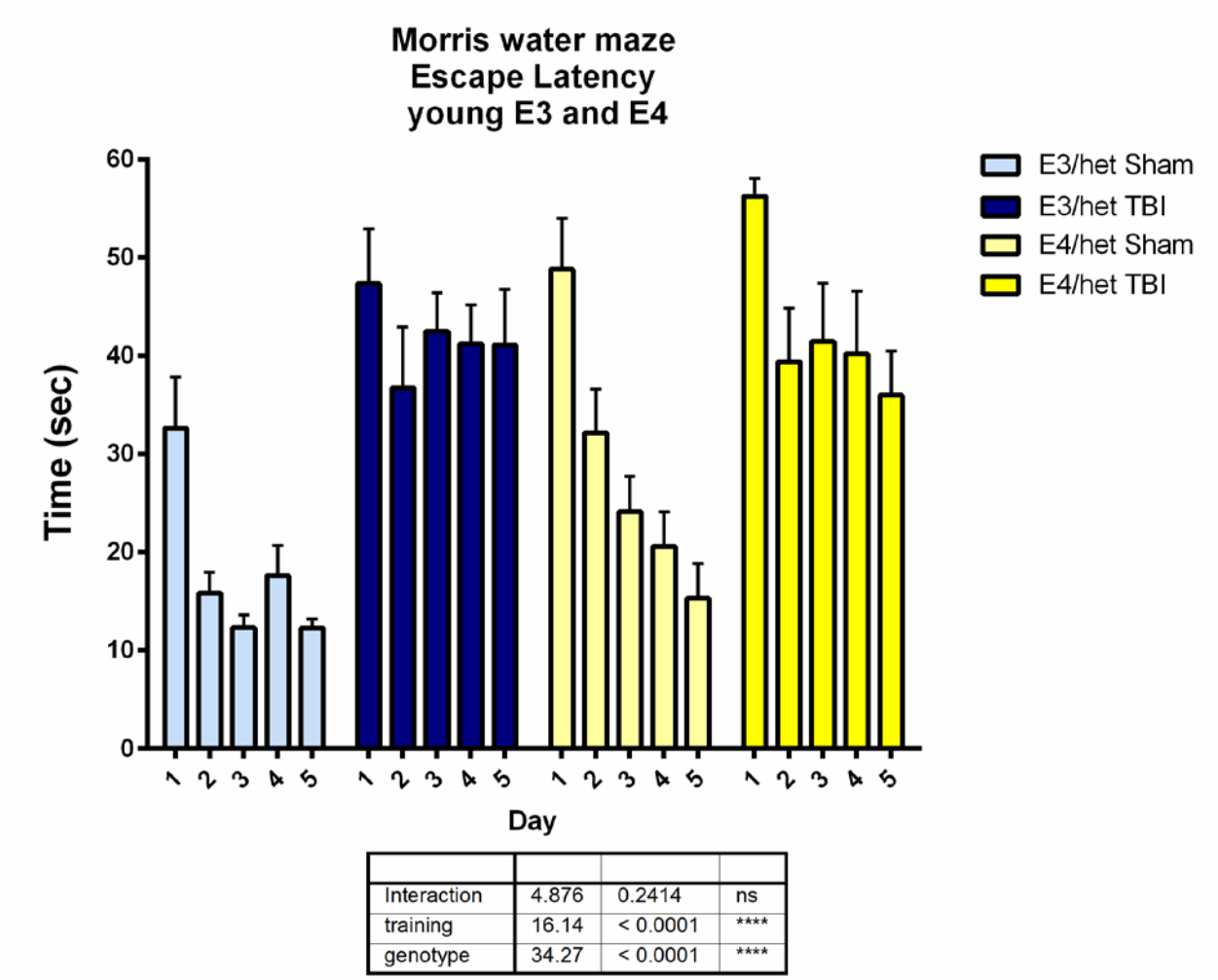
- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - Nothing to report
- **What other organizations were involved as partners?**
  - Nothing to report

8. **FIGURES**



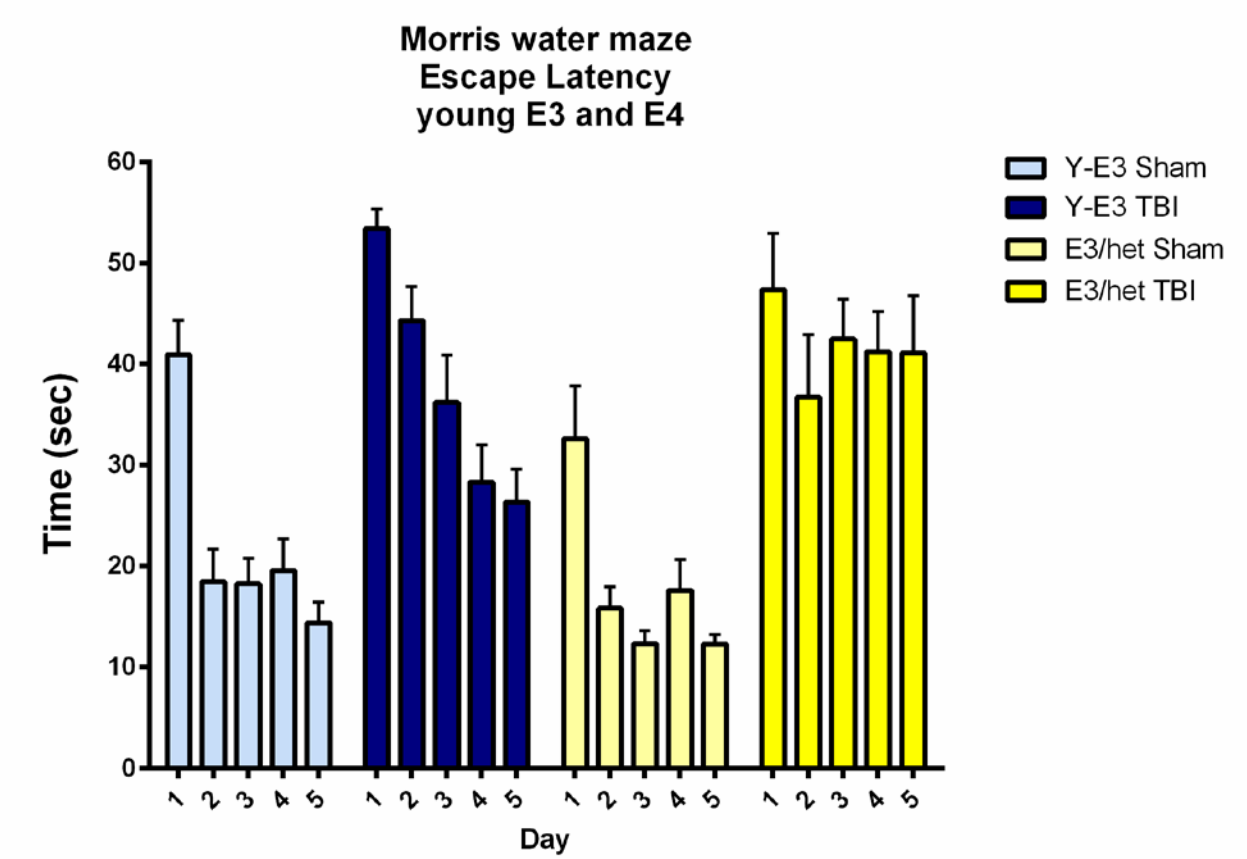
Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	2.192	0.3667	ns	No
Genotype	5.495	0.0516	ns	No
Injury	27.72	< 0.0001	****	Yes

Figure 1: Traumatic brain injury significantly increases time spent in the open arms of elevated plus maze in Abca1 deficient mice. At 4DPI, we tested all mice in the EPM for anxiety-related behaviors and analyzed their behavior. TBI mice in all genotypes spent significantly more time in the open arms of the elevated plus maze ($p<0.0001$) when measured by 2-way ANOVA. Additionally, the effect of genotype on anxiety levels is trending ($p<0.0516$). Both groups on human APOE4 background had significant differences between sham and TBI as determined by post-hoc testing.



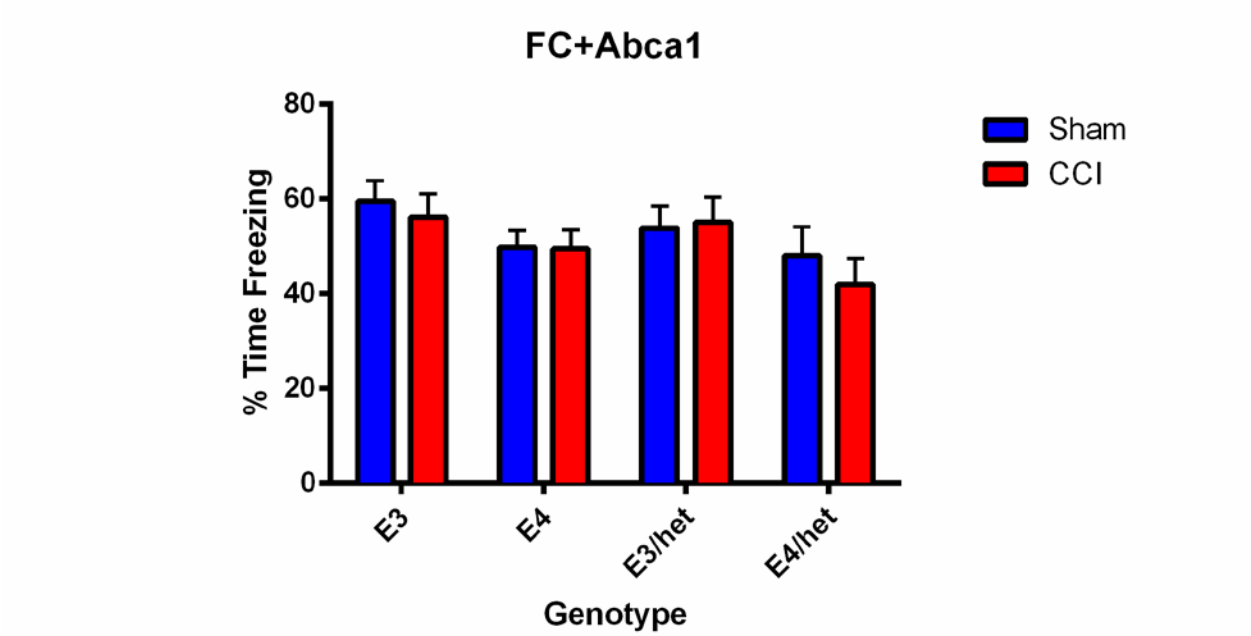
Interaction	4.876	0.2414	ns
training	16.14	< 0.0001	****
genotype	34.27	< 0.0001	****

Figure 2: Traumatic brain injury significantly increases cognitive deficits as measured with Morris Water maze in Abca1 deficient mice. Mice were trained to develop a spatial association between visual cues and a hidden platform on days 7-11 post-injury. Time (mean + SEM) to find the hidden platform is shown by group for all 5 days of training. There was no interaction seen between injury/genotype and training day performance in the either genotype, as determined by Two-way Anova, however performance was significantly affected by genotype/injury ($p<0.0001$) and training day ($p<0.00001$). Injury in Abca1 deficient animals display minimal display minimal learning following training, as seen in Abca1 wt animals previously.



Interaction	5.239	0.0406	*
training	16.25	< 0.0001	****
genotype	32.89	< 0.0001	****

Figure 3: Abca1 deficiency interacts with traumatic brain injury to significantly affect performance in Morris Water maze on APO E3 background. Mice were trained to develop a spatial association between visual cues and a hidden platform on days 7-11 post-injury. Time (mean + SEM) to find the hidden platform is shown by group for all 5 days of training. There is a significant interaction between genotype/injury and training ($p<0.05$), in addition to genotype/injury and training both significantly affect performance in Morris Water maze ($p<0.0001$). Abca1 deficiency in addition to the human APOE3 background leads to minimal learning over the course of training days.



Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	0.7071	0.8961	ns	No
Genotype	9.701	0.0489	*	Yes
Injury	0.4385	0.5438	ns	No

Figure 4: Genotype significantly affects cognitive performance in fear conditioning, not traumatic brain injury. On 13 and 14DPI mice were trained with to learn an association between their environment and a foot shock, and then we tested their ability to remember this context. Genotype, but not injury, significantly affected freezing response during this paradigm ($p<0.05$).

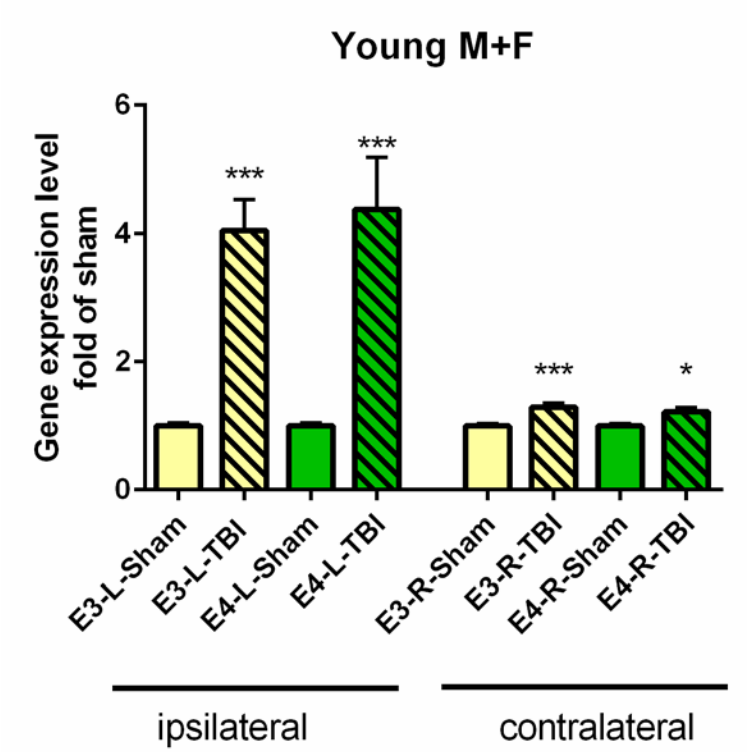


Figure 5: Traumatic brain injury significantly increases gene expression of Trem2. In both the ipsilateral and contralateral hemispheres of injured animals, there is a significant difference in gene expression of Alzheimer's related gene, Trem2 ($p<0.05$). Gene expression was measured using RT-qPCR and values shown are fold of sham.

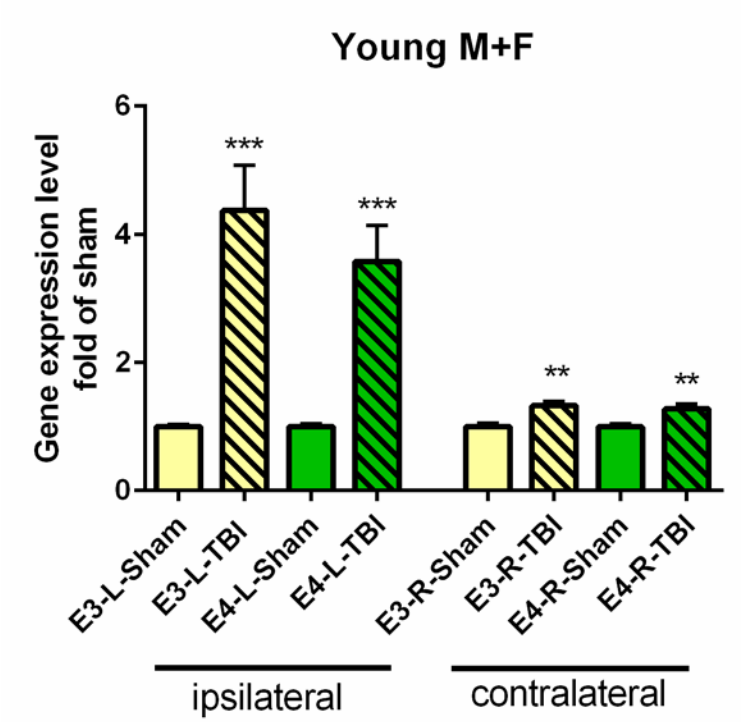


Figure 6: Traumatic brain injury significantly increases gene expression of Abca1. In both the ipsilateral and contralateral hemispheres of injured animals, there is a significant difference in gene expression of Abca1 ($p<0.05$). Gene expression was measured using RT-qPCR and values shown are fold of sham.

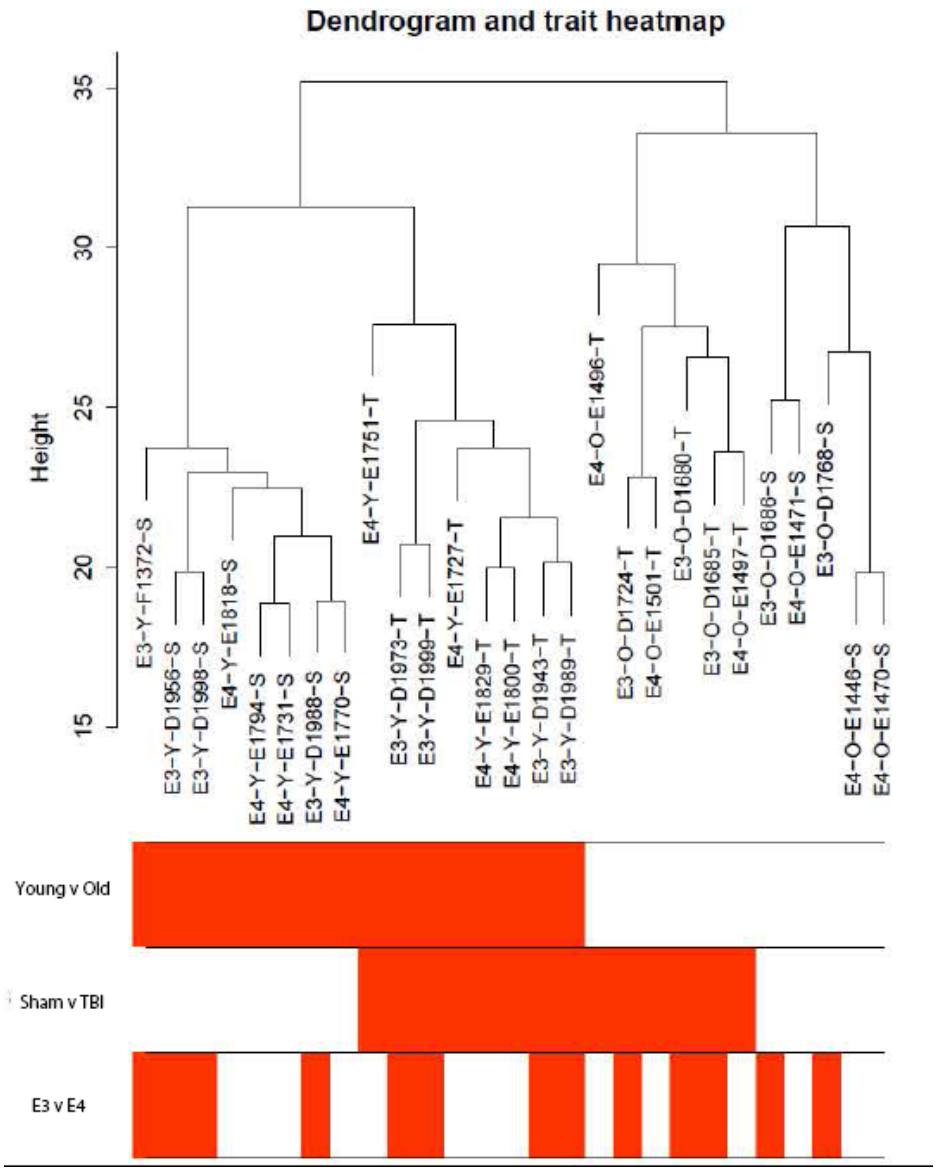


Figure 7: Clustering dendrogram of samples based on their Euclidean distance with a color indication of trait. The dendrogram shows no obvious outliers. Two traits as shown in colored bars below the dendrogram coincide with branching, which suggests that Injury and Age are globally distinct within the separated groups. The color demonstrating the ApoE trait does not coincide with branching, so it is not a factor affecting separation of animals. **Red color** indicates 1 factor of each trait; young, injury and E3; **white color** indicates the other possible trait characteristic: old, Sham or E4. Each animal is assigned 1 factor per trait, but the traits do not exclude each other.

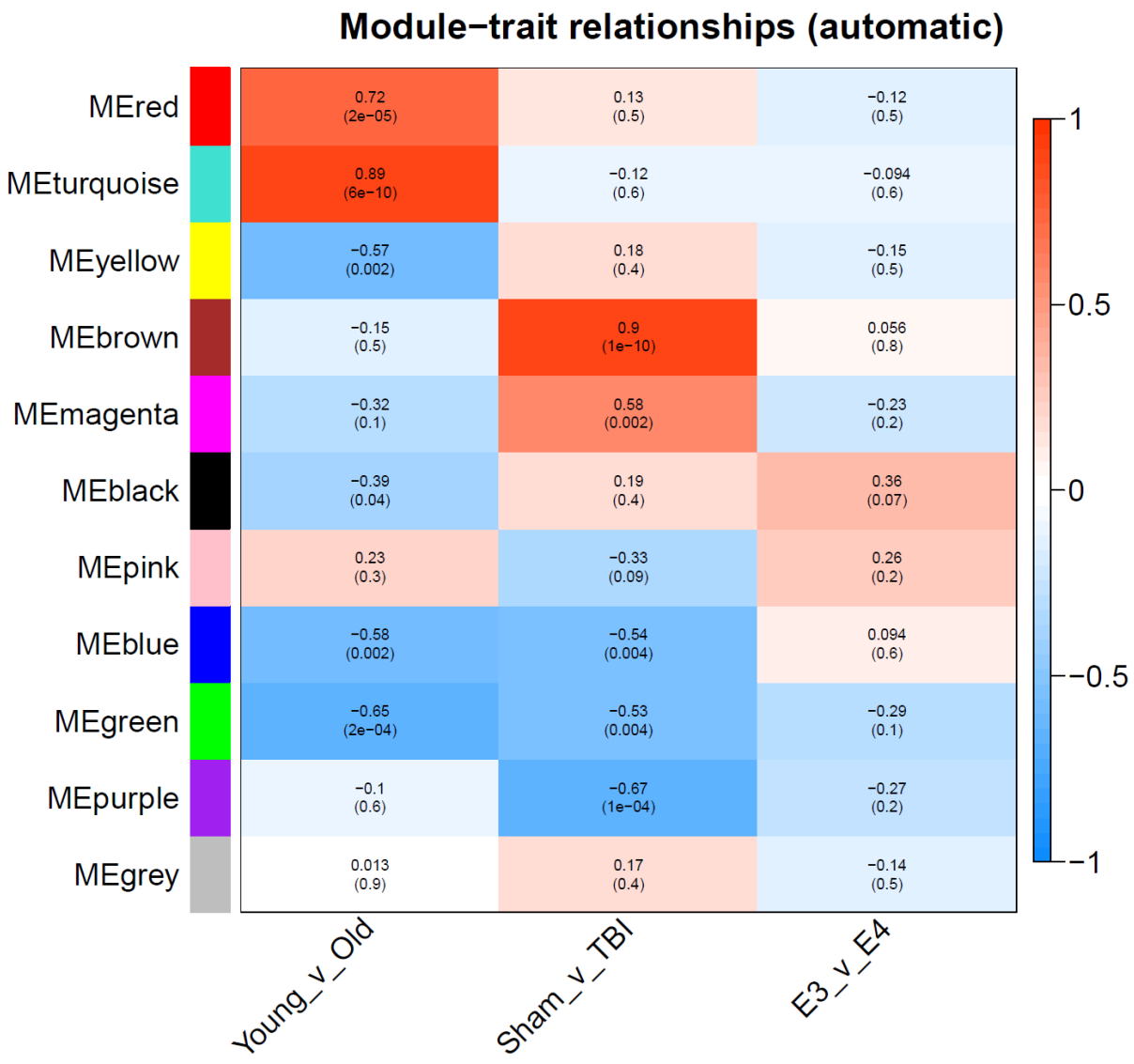


Table 1: Module-trait associations. Each row corresponds to a module eigengene, each column corresponds to a trait. Each cell contains the corresponding correlation and p-value. The table is color-coded by correlation according to the color legend on the right. Modules of interest are differentially expressed between trait conditions. Brown and magenta are upregulated modules associated with TBI, red and turquoise are associated with age. Purple is a downregulated module associated with TBI, yellow and black with age. Blue and green are downregulated in both TBI and age. These modules are also significant.

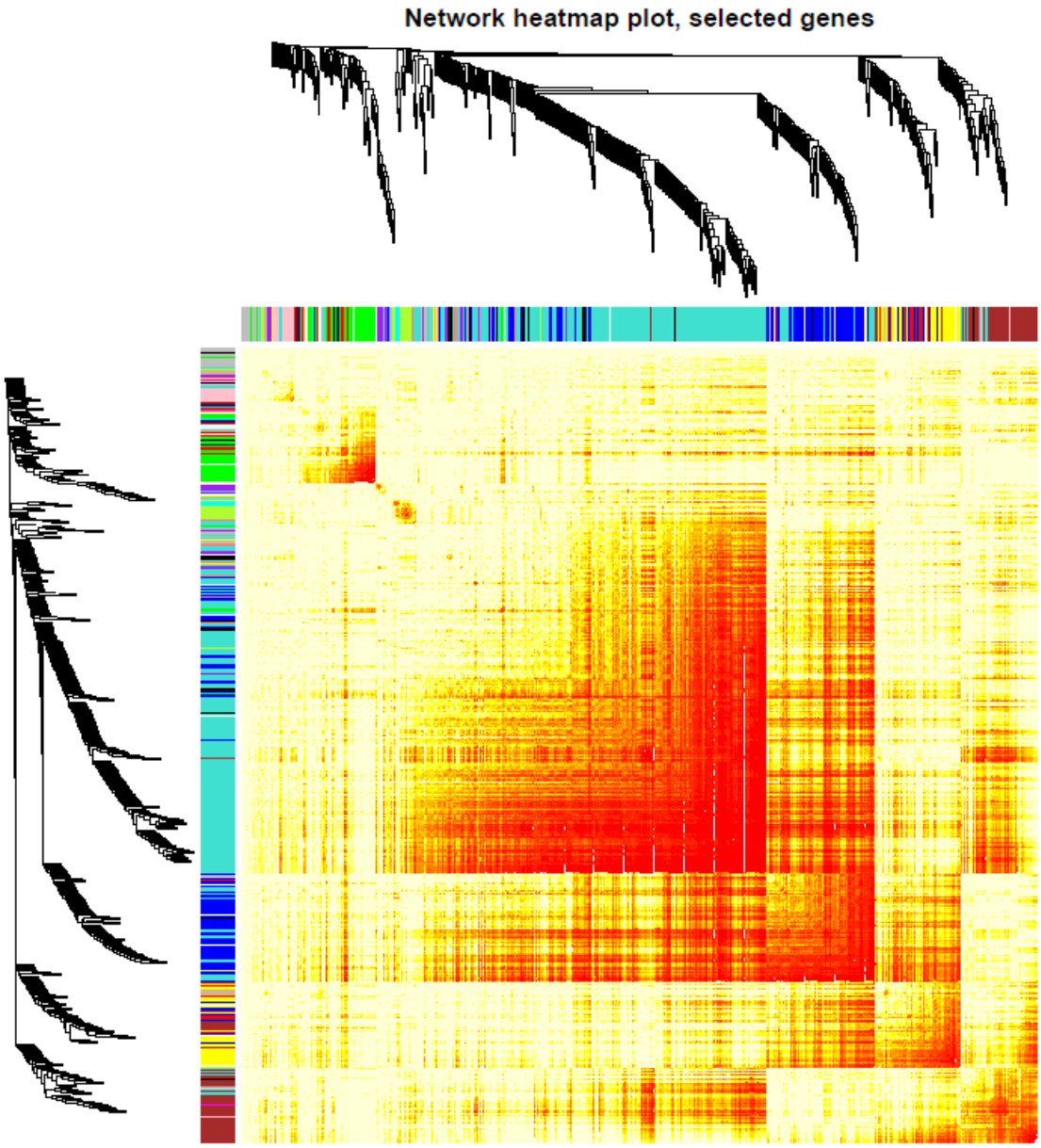
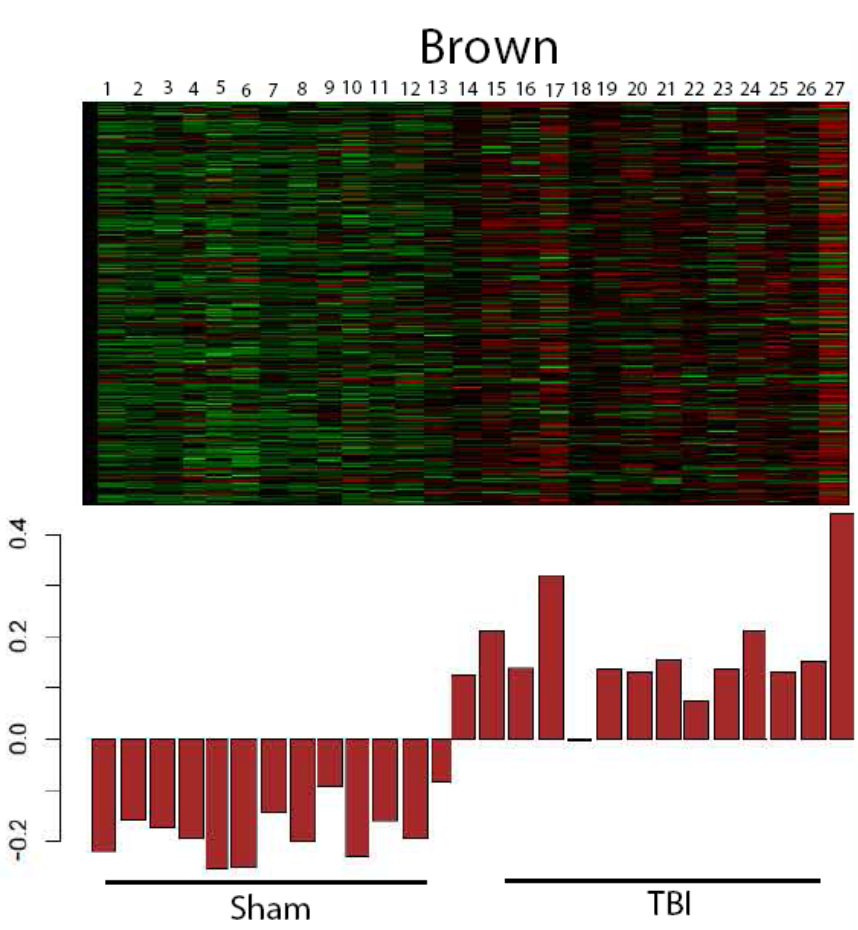


Figure 8: Visualizing the gene network using a heatmap plot. The heatmap depicts the overlap among the top 500 genes in the modules. Each row and column corresponds to a single gene, with increasing overlap being signified by darker color. The higher the overlap between two genes signifies increasing likelihood that the genes belong to the same family of biological function. Blocks of color along the diagonal represent a module, a group of genes with high overlap or strong interactions. The gene dendrogram and module assignment are shown along the left side and top.



Sham v TBI				
D1956	E3	Sham	Young	1
D1988	E3	Sham	Young	2
D1998	E3	Sham	Young	3
F1372	E3	Sham	Young	4
E1818	E4	Sham	Young	5
E1794	E4	Sham	Young	6
E1770	E4	Sham	Young	7
E1731	E4	Sham	Young	8
D1686	E3	Sham	Old	9
D1768	E3	Sham	Old	10
E1446	E4	Sham	Old	11
E1470	E4	Sham	Old	12
E1471	E4	Sham	Old	13
D1943	E3	TBI	Young	14
D1973	E3	TBI	Young	15
D1989	E3	TBI	Young	16
D1999	E3	TBI	Young	17
E1829	E4	TBI	Young	18
E1800	E4	TBI	Young	19
E1751	E4	TBI	Young	20
E1727	E4	TBI	Young	21
D1724	E3	TBI	Old	22
D1685	E3	TBI	Old	23
D1680	E3	TBI	Old	24
E1501	E4	TBI	Old	25
E1497	E4	TBI	Old	26
E1496	E4	TBI	Old	27

Figure 9: Module heatmap of gene expression with corresponding sample module eigengene (ME) value. Within the heatmap, the rows correspond to genes and the columns to samples; green denotes underexpression and red denotes overexpression. Below are shown the corresponding module eigengene values for each sample. The ME can be considered the most representative gene expression profile of the module. The ME values and gene expression values denote that these genes are underexpressed in sham animals and overexpressed in TBI.

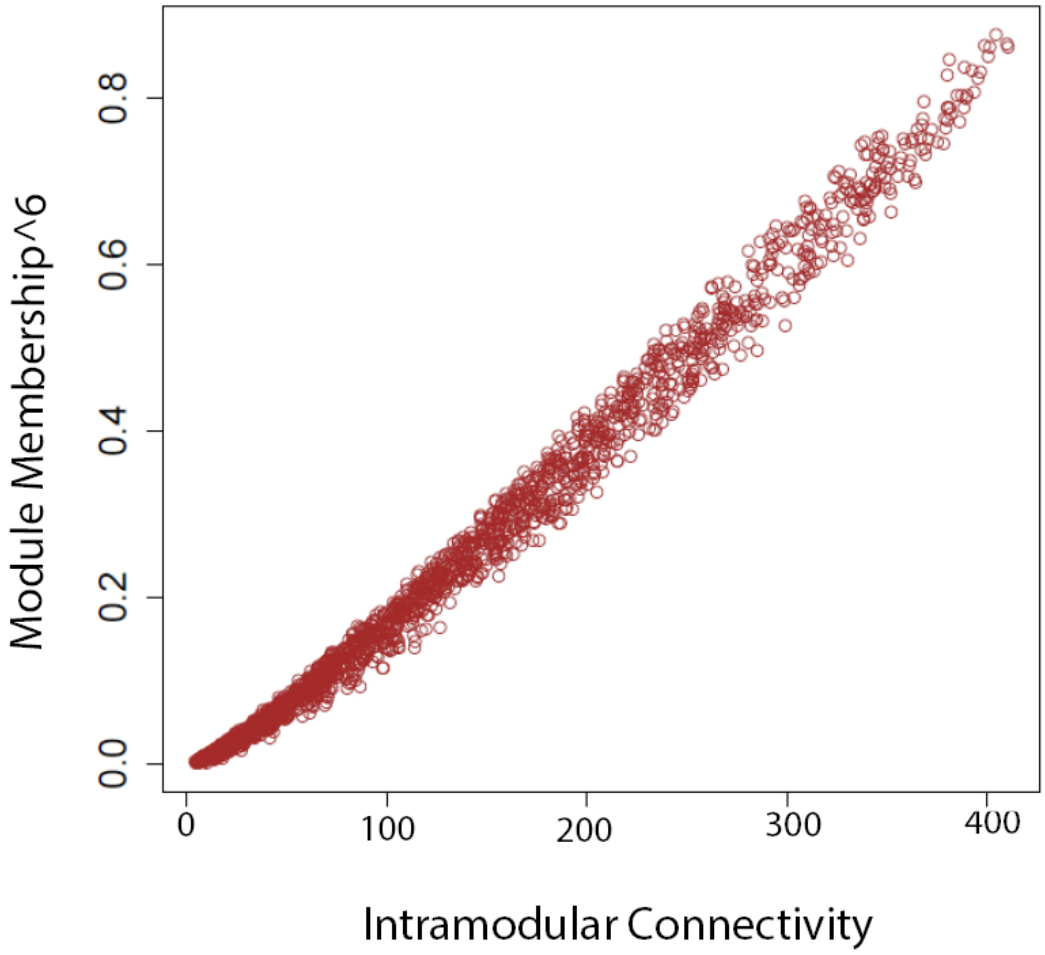


Figure 10: Module membership correlated to intramodular connectivity for Hub gene identification. Module eigengene is considered the most representative gene expression profile for all the genes present within the network. The strength of each genes expression to correlate with that of the module eigengene is its module membership value. Genes most biologically implicated within the module are going to have a high module membership value and strongly connect to other genes within the network. The figure visualizes the relationship of these two factors, with Hub genes being the genes with the highest values on the correlation. A default cutoff of 0.8 was used to identify hub genes. Shown here is the correlation for the brown module ($R = 0.99$, $p<1e\text{-}200$). There were 51 hub genes identified within this module, including Clu, Tyrobp and Axl.

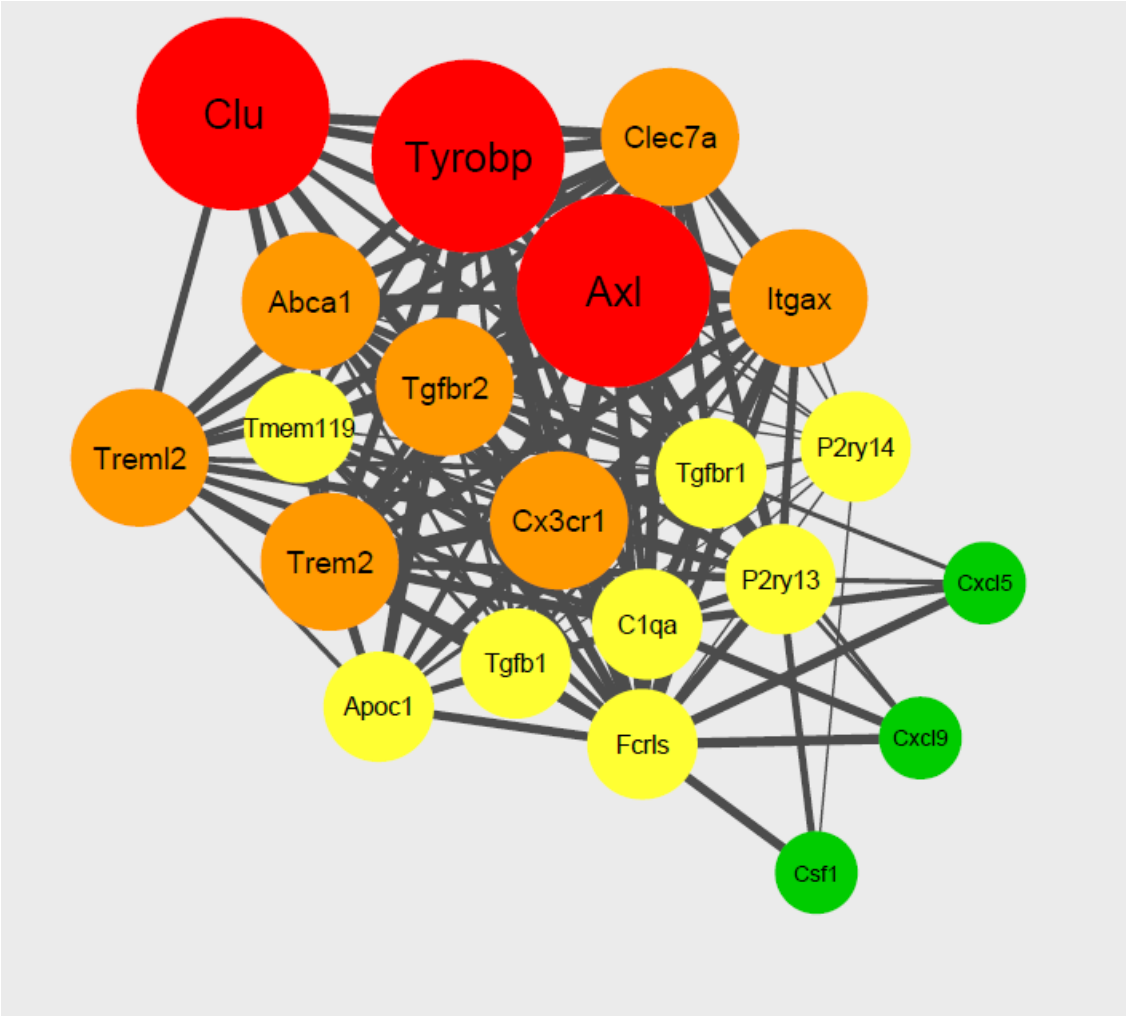


Figure 11: Visualization of brown module network. Following identification of hub genes, the hub genes are compared with the directionality of the module. Since the brown module is overexpressed in TBI, we were most interested in hub genes also overexpressed in TBI. The hub genes were then narrowed down more based on relationship to the module annotation using GO enrichment. Hub genes were chosen based on its biological function relationship to "immune response" and "inflammatory response". In this network, Clu, Tyrobp and Axl were chosen as the hub genes. The targets of the hub genes and the targets of the hub gene targets demonstrate the biological function of the module and the connectedness of these genes within the module.

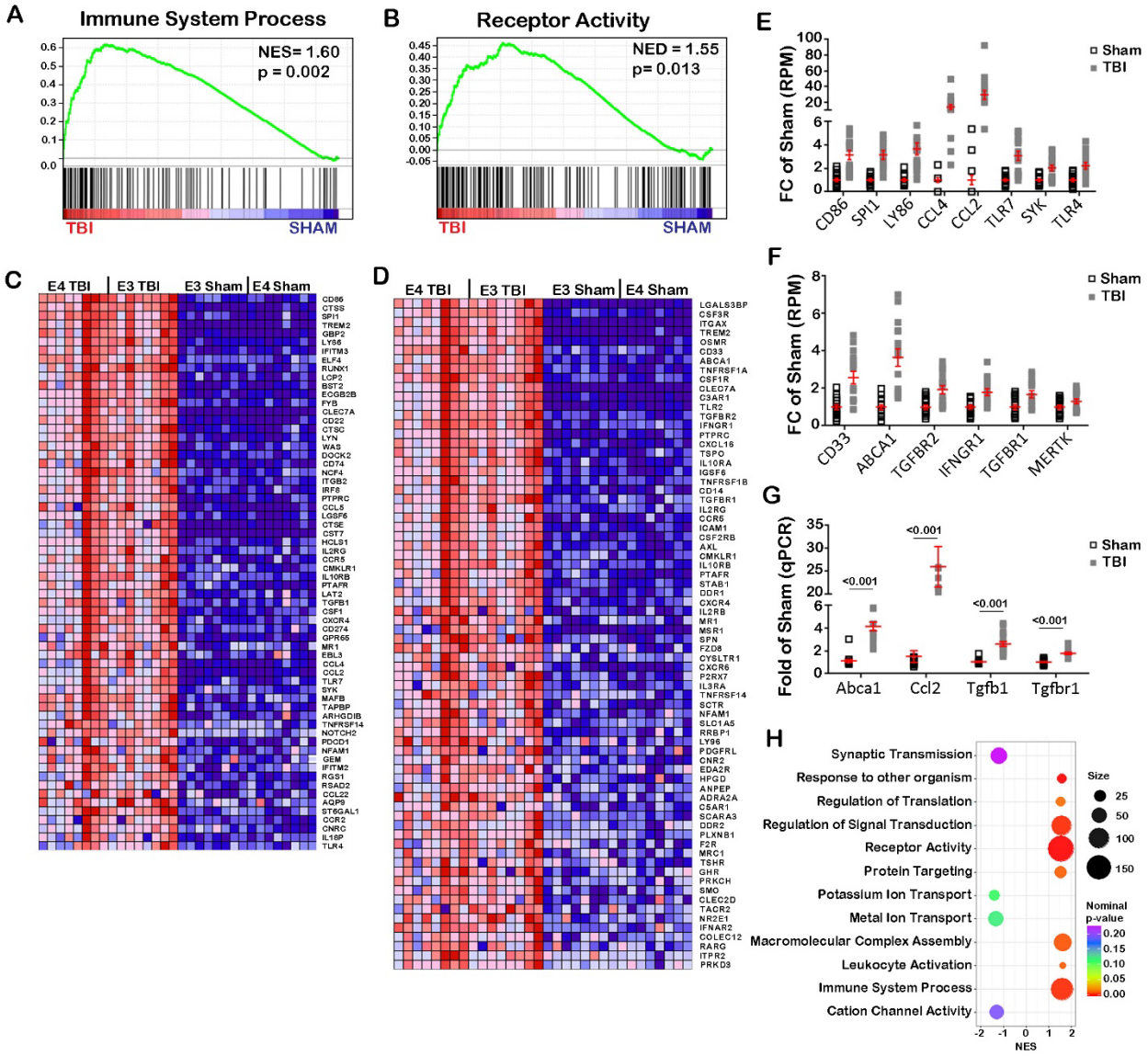


Figure 12: Differential expression and Gene set enrichment analysis 14 days post – injury. Compared are all Sham mice versus TBI mice, APOE3 and APOE4 isoforms combined within each group. GO terms significantly enriched in injured mice for (**A,C**) "Immune System Process" and (**B,D**) "Receptor Activity" at p<0.05. GSEA enrichment scores curves (**A-B**) and heatmaps (**C-D**) provided by the GSEA analysis are shown for the respective GO terms. The upregulated genes are represented in red and downregulated genes are represented in blue. (**E**) RNA-seq results for selected genes from the GO term "immune system process" are shown as normalized to the average of sham. (**F**) RNA-seq results for selected genes from the GO term "receptor activity" are shown as normalized to the average of sham. (**G**) Validation of selected genes from both GO terms by RT-qPCR is shown. Statistics were determined by t-test $p<0.05$. (**H**) The bubble plot shows top ranked GO terms affected by the injury. Color indicates normalized p value and size of bubble indicates the number of genes assigned to the GO term.

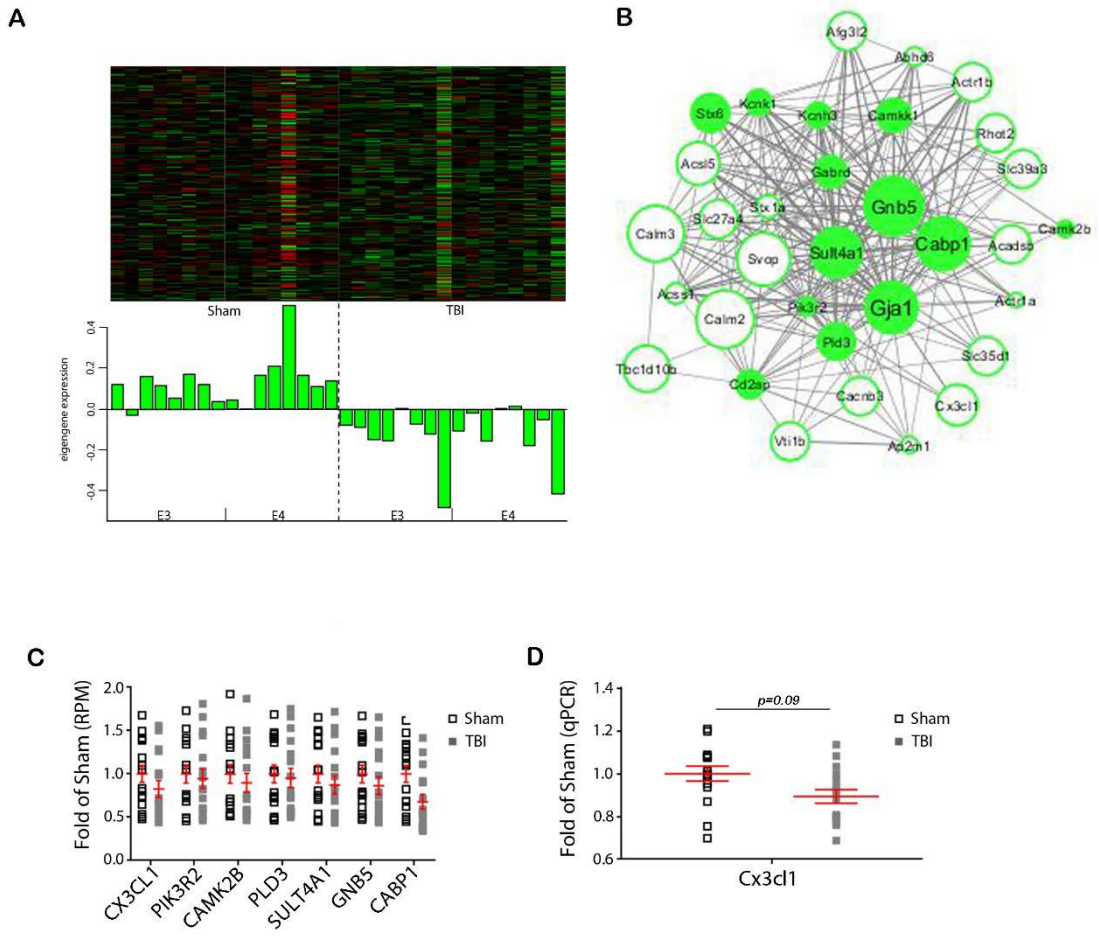


Figure 13: Gene-network module green is downregulated in TBI animals and reflects biological process "transport". The green module is downregulated in correlation with TBI. (**A**) The expression barplot shows the gene expression and eigengene expression within each sample. (**B**) Network of genes connected to hub genes *Pik3r2* and *Camk2b.* (**C**) RNA-seq results for important genes within the network. The average expression was calculated as fold of Sham reads per million for each gene and sample to which the TBI animals are normalized. Statistics is by edgeR, $p<0.05$. (**E**) Validation of RNA-seq results by RT-qPCR. Statistics was determined by t-test.

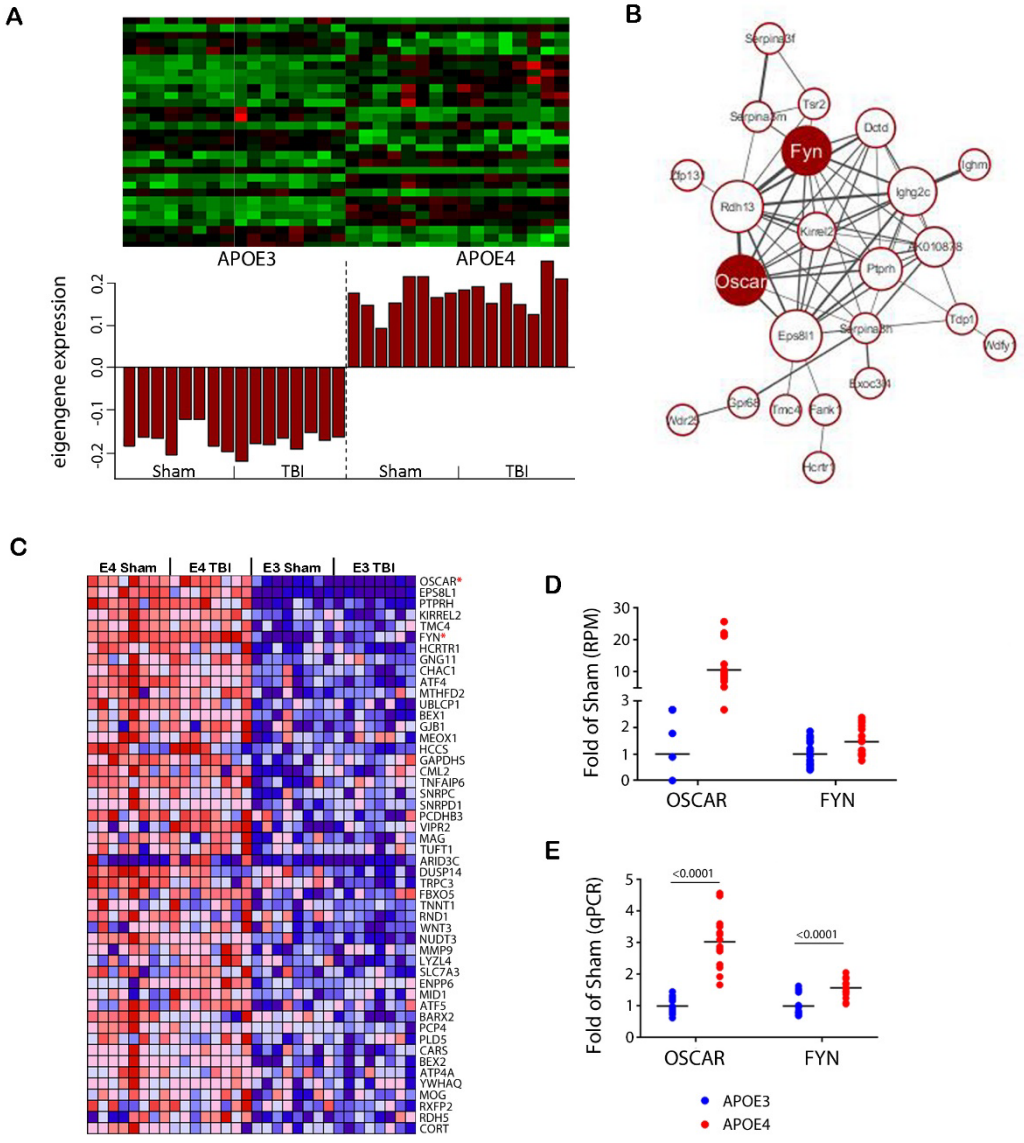


Figure 14: Gene-network module darkred is modulated by APOE isoform and reflects biological process "innate immunity". The "darkred" module is upregulated in correlation with APOE4 isoform. (**A**) The expression barplot shows the gene expression and eigengene expression within each sample. (**B**) Network of genes connected to hub genes *Fyn* and *Oscar* representing innate immunity. (**C**) Heatmap of top 50 upregulated genes in comparing APOE3 to APOE4 mice. (**D**) RNA-seq results for important genes within the network. The average expression was calculated as fold of Sham reads per million for each gene and sample to which the TBI animals are normalized. Statistics is by edgeR, $p<0.05$. (**E**) Validation of RNA-seq results by RT-qPCR. Statistics was determined by t-test.

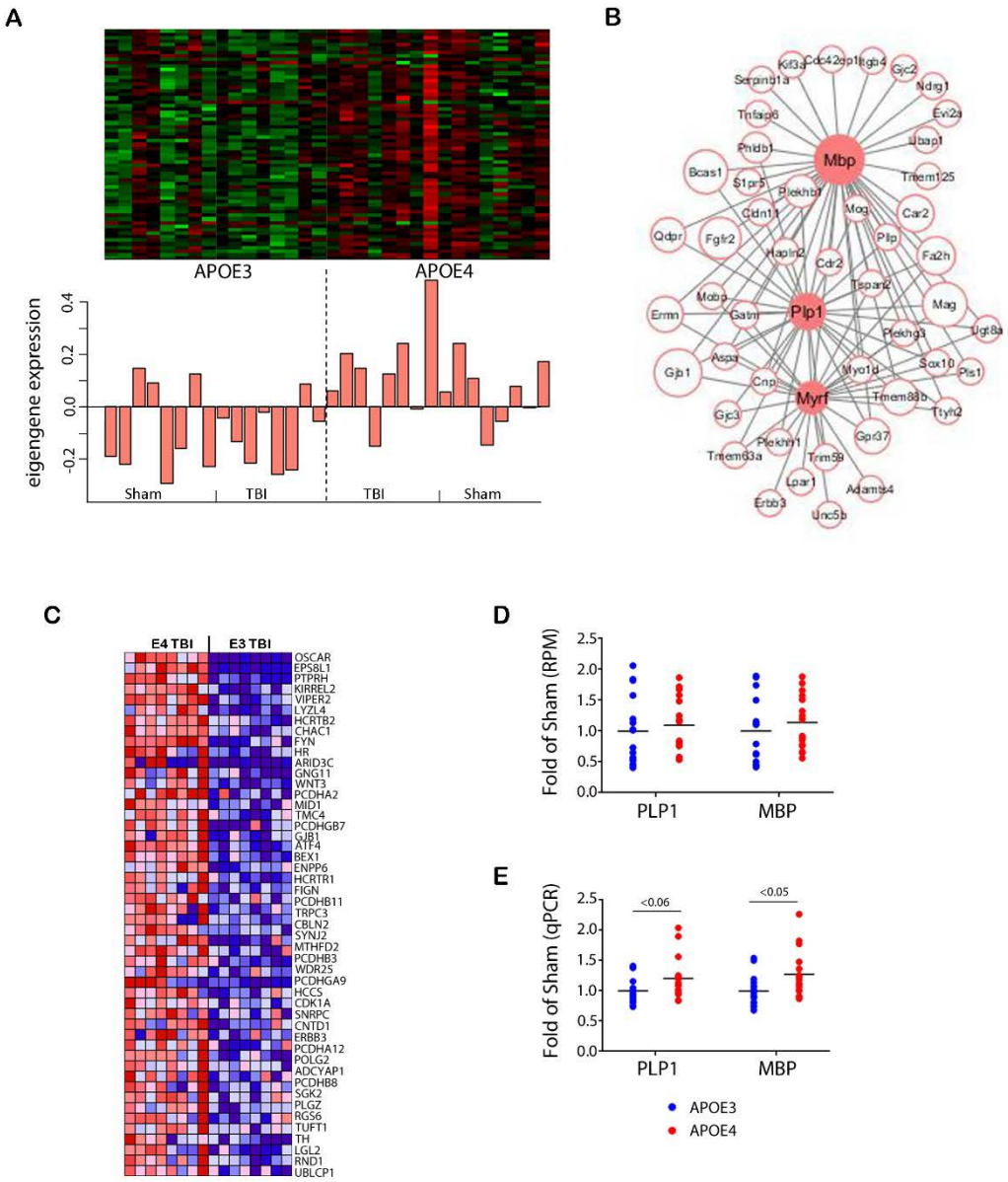


Figure 15: "Myelination" module. The salmon module is upregulated in correlation with APOE isoform and TBI. (**A**) The expression barplot shows the gene expression and eigengene expression within each sample. (**B**) Network of genes connected to hub genes *Plp1* and *Mbp* representing myelination. (**C**) Heatmap of top 50 upregulated genes in comparing APOE3 TBI to APOE4 TBI mice. (**D**) RNA-seq results for important genes within the network. The average expression was calculated as fold of Sham reads per million for each gene and sample to which the TBI animals are normalized. Statistics is by edgeR, $p<0.05$. (**E**) Validation of RNA-seq results by RT-qPCR. Statistics was determined by t-test.

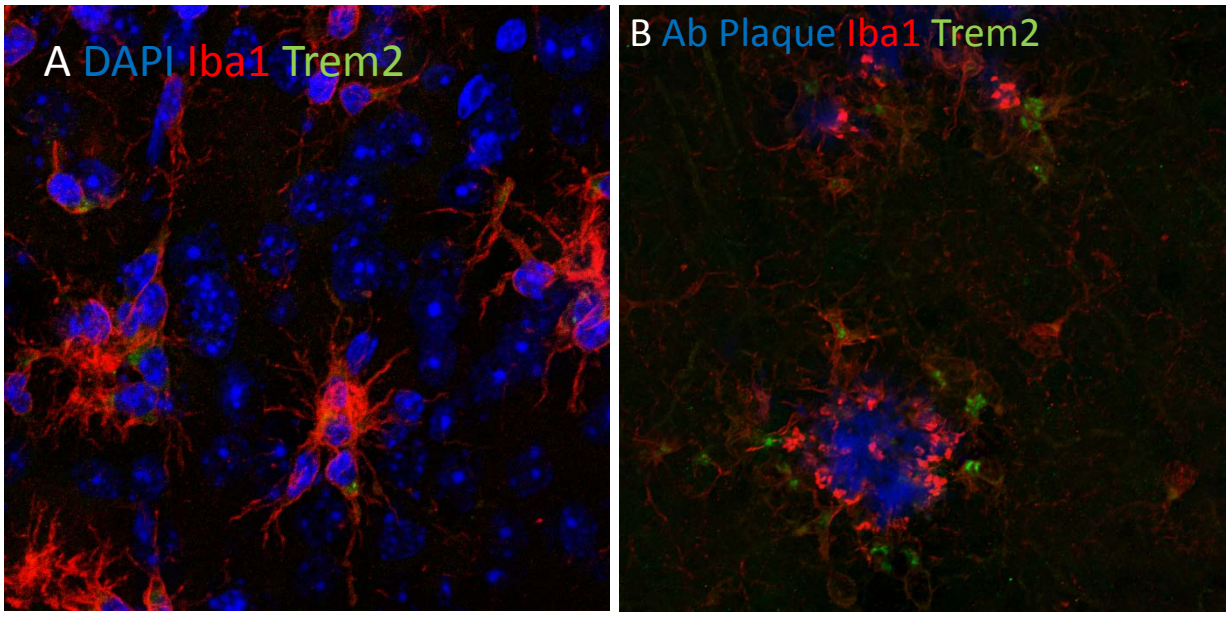


Fig. 16: Trem2 Immunohistochemistry in APP mice. Protocol for Trem2 fluorescent staining has been optimized in APP mice. (**A**) Image shows Trem2 (green) and Microglia (red) co-localization. Nuclei are stained with DAPI. (**B**) Image shows amyloid plaque deposits in APP mice cortex are surrounded by microglia (red) that are expressing Trem2 (green).

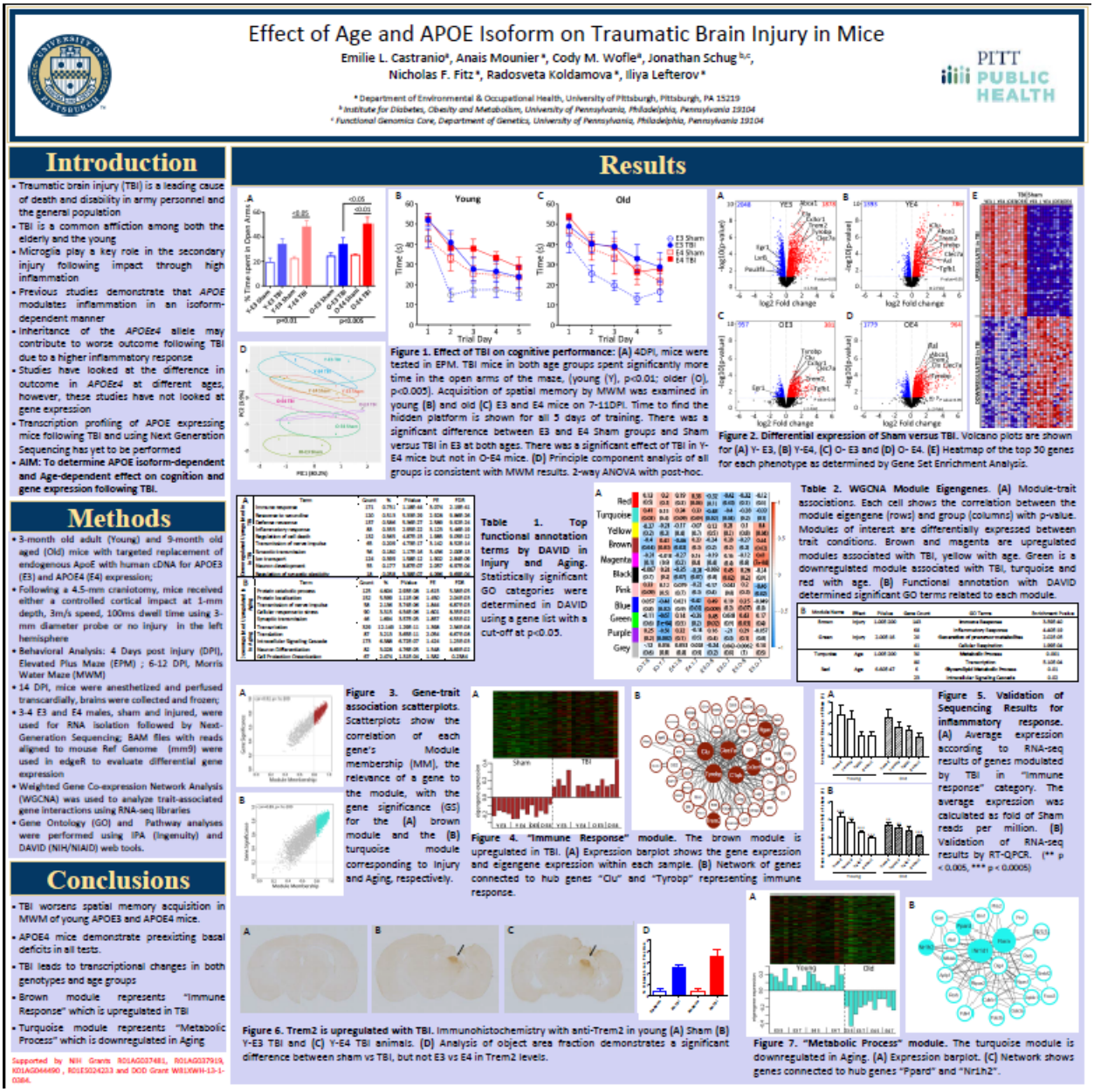


Figure 17: Poster for Keystone Symposium

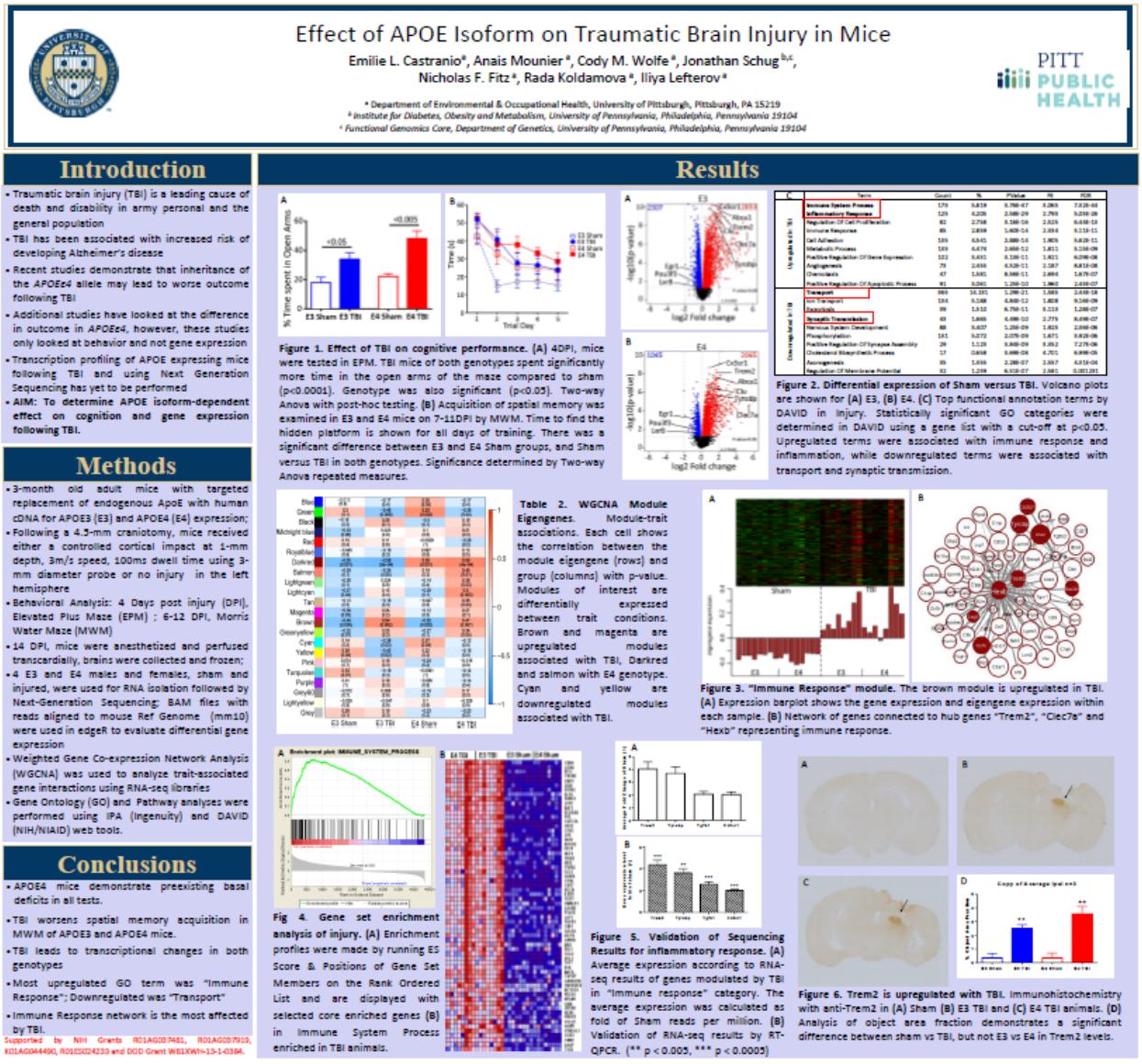


Figure 18: Poster for Gordon Conference

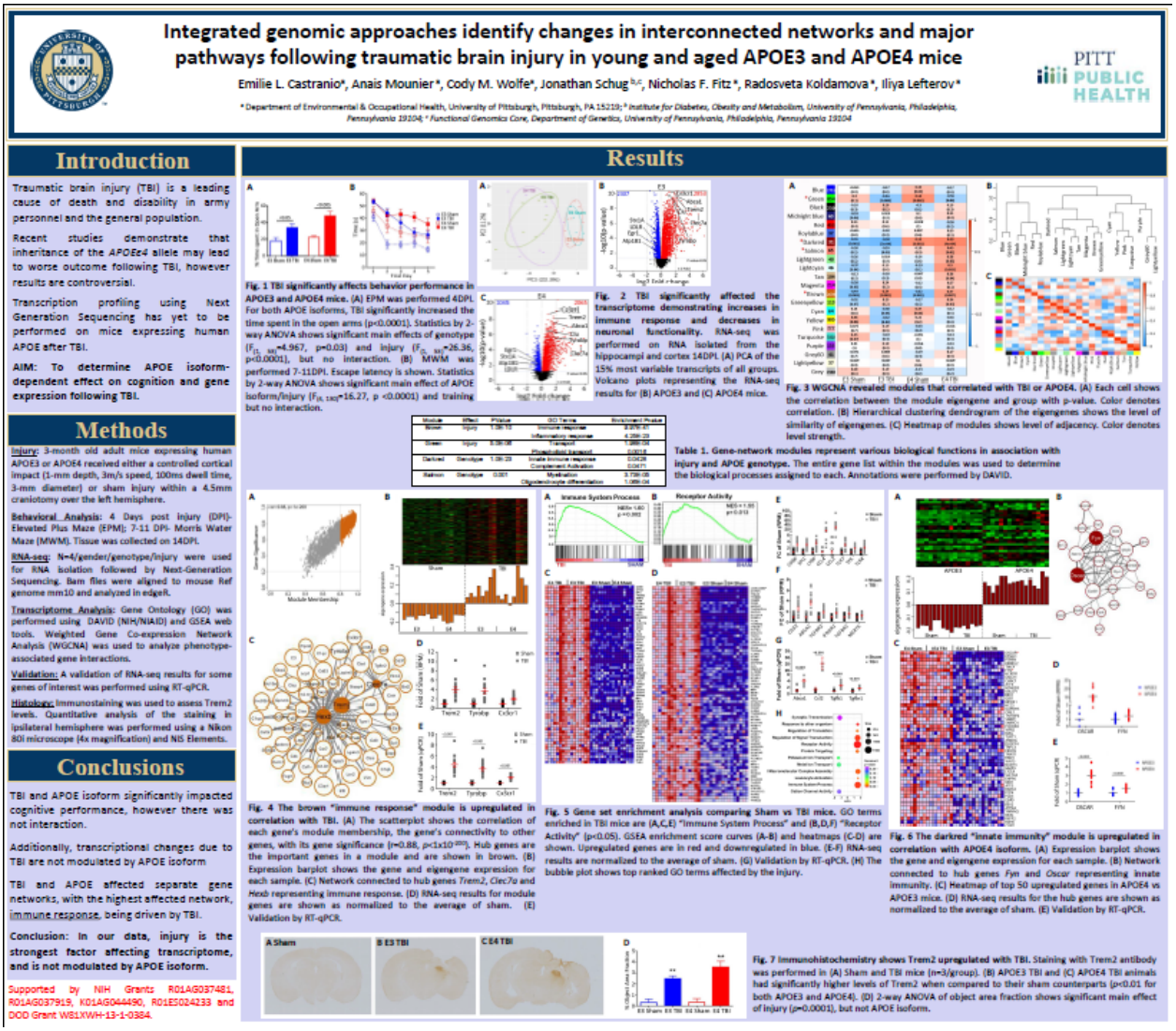


Figure 19: Poster for Society for Neuroscience